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14. ABSTRACT

The adhesion receptor CD44 is known to decrease the metastatic potential of breast cancer cells in vivo. This study focuses on understanding the mechanisms by which CD44 inhibits breast cancer cell invasion. We have found that the differential interaction between CD44 and soluble or embedded Hyaluronan leads to differential phosphorylation of ERK and Pak. These differentially activated signaling pathways molecularly govern a phenotypic switch between invasiveness or proliferation. Additionally, this interactions also leads to decreased transcription of the metalloprotease MMP9 when on collagen IV gels but not collagen I gels. Together, these mechanisms provide significant insight into how CD44 inhibits the movement of breast cancer cells away from their primary site.

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Introduction

The Interactions that occur between tumor epithelial cells and the surrounding stroma are important in determining the disease progression of various cancers, including breast cancner. Recent studies have found that the interactions between the epithelial adhesion receptor, CD44, and the extracellular sugar, hyaluornan (HA) can decrase the tumor epithelium's ability to invade and metastasize. Our research is aimed at elucidating the mechanisms by which CD44-HA interactions lead to decreased tumor invasion.

BODY
SECTION 1. DISTINCT INVASIVE OR PROLIFERATIVE PHENOTYPES ARE DEPENDENT UPON HYALURONAN PRESENTATION

Metastatic disease is the leading cause of mortality among breast cancer patients and it is evident that changes occur both within epithelial cells as well as the surrounding stroma to accommodate invasion. Mammary epithelial cells must lose polarity, detach from neighboring cells and gain motility before they can metastasize to distant organs. However, the tissue surrounding the tumor epithelium is of equal importance as tumor progression is clearly dependent on angiogenesis, immune cell evasion and the composition of the extracellular matrix [1-3]. Under normal conditions, the basement membrane is responsible for maintaining the tissue architecture upon which the epithelium rests, and for providing a clear boundary that isolates the epithelium from the underlying connective tissue. During breast cancer progression, however, a crosstalk is established between the tumor epithelium and tumor associated fibroblasts that often leads to a desmoplastic reaction, resulting in a dense extracellular matrix encapsulating the tumor [5]. Desmoplasm may change based on the composition of the matrix which can be comprised of a variety of ECM components including fibronectin, collagen IV, thrombospondin-1, transforming growth factor-β and hyaluronan (HA) [1].

HA is a disaccharide ubiquitously expressed throughout extracellular matrices. It binds the adhesion receptor CD44, affecting multiple processes such as migration, proliferation, angiogenesis, apoptosis and adhesion[6, 7]. The effects of HA/CD44 interactions on mammary tumor progression remain controversial due to conflicting observations. In breast cancer, increased serum HA is observed in women with metastatic disease and is an indicator of poor disease outcome [8]. HA is deposited stromally by resident fibroblasts in the mammary gland, with increased HA deposition observed as tumors progress[9-12]. In addition, studies demonstrate that HA production by epithelial cells lead to increased invasion and metastasis [13]. However, recent studies indicate that HA catabolism and the generation of HA oligos, along with loss of epithelial CD44 can facilitate tumor progression[14-17]. Alternatively, increased expression of CD44 in breast tumors correlate well with patient survival [18, 19] and is commonly associated with tumor types that rarely metastasize[20].

Recently, we observed that loss of CD44 in the MMTV-PyV mt mouse model of breast cancer led to a 6-fold increase in metastasis, indicating that CD44-HA interactions can protect from mammary tumor metastasis[12]. In addition, breast cancer cells were found to invade into collagen I gels with less efficiency when hyaluronan is cast as part of the matrix. Importantly, mice expressing GFP under the control of the Has2 promoter, the primary synthase for HA, displayed extensive deposition of HA in the stromal compartment of tumors, but not in tumor epithelium [12]. These studies demonstrate that HA is primarily produced by cells of the matrix, and that this matrix deposited stroma corresponds with an inhibition of invasion and metastasis. Yet studies in traditional 2 dimensional (2D) culture systems continue to point to a pro-invasive role for HA and CD44[7, 12, 21, 22].

In the present study, we designed experiments to determine the mechanism by which CD44 interactions with HA can alter cell invasion into a stromal-like matrix. We now present data demonstrating that transformed cells grown on a collagen matrix behave differently depending on whether HA is present in the extracellular matrix (eHA) or presented in a soluble form (sHA). We find that cells encountering collagen matrices with eHA show delayed cellular spreading after seeding, decreased invasion, migrate mainly through the use of lamellipodia and have enhanced proliferation. In contrast to this, cells that encounter collagen with sHA in their environment have increased levels of invasion, and decreased proliferation. The addition of this simple sugar to the cellular microenvironment has profound effects on cellular phenotypes that depend on its presentation. This study may have significant implications on how we understand matrix components to function and the role of their presentation in the cellular microenvironment.

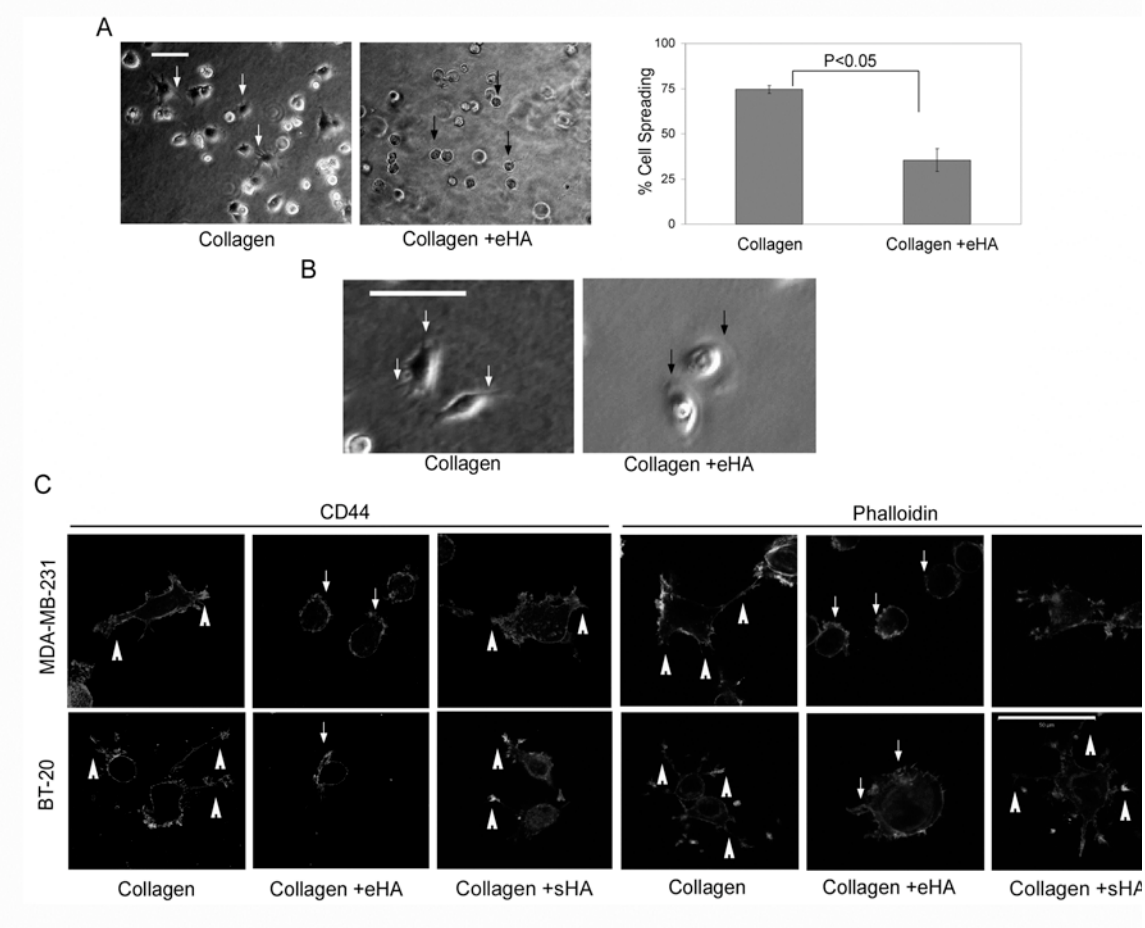

Figure 1.1 Cell morphology changes dependent on HA in collagen I gels.
(A) MDA-MB-231 cells ($5x10^4$) were placed onto 1mg/ml type I collagen gels polymerized in the presence or absence of 75μg/ml HA. 45 minutes after placement of cells on matrix, cells were imaged and spreading was quantified. Statistics were calculated using an unpaired student's t-test. The error bars represent s.d. of the entire gel from 3 separate experiments. (B) Time lapsed video microscopy was obtained from cells seeded as described in **a** over 1.5 hrs, revealing filopodia formation (left panel, white arrow) when on collagen and lamellipodia formation (right panel, black arrow) when on collagen + eHA. (C) CD44 and actin localization was determined using CD44 antibodies and phalloidin (DF1485 and FITC-phalloidin). Filopodia formation (white arrowhead) was observed in collagen gels + eHA (right panels) and lamellipodia were observed in collagen gels without HA (left panels). Scale bar indicates 25 μm.

Results

Cells alter adhesions when HA is cast as part of a collagen matrix

Previous work has identified the stromal compartment surrounding the tumor epithelium as the main repository of HA and HA producing cells, indicating that CD44 function must be studied in the context of epithelial-stromal interactions [9, 12, 23-25]. Utilizing three separate metastatic breast cancer cell lines (MDA-MB-231, BT20 and MD-MB-468), we have previously shown *in vitro* that epithelial CD44 interacts with collagen-embedded HA to inhibit invasion[12]. To evaluate the mechanism by which collagen bound HA inhibits invasion, we observed the adhesion dynamics of cells adhering to a matrix.

To analyze adhesion, MDA-MB-231 breast cancer cells were observed over a three hour period after seeding on collagen I gels with (eHA) or without high molecular weight HA (avg. 2MDa). Cells were seeded onto gels and visually monitored for adhesion and spreading. While all cells adhered to the surface of the gels equally, cells that encountered eHA demonstrated a significant delay in spreading onto the gels (Figure 1.1A, right panel, black arrows). In the absence of eHA, almost 75% of cells seeded onto the collagen matrix attached and spread on the gel by 45 minutes (Figure 1.1A, left panel, white arrows). Alternatively, only 35% of cells adhered to eHA collagen matrices (Figure 1.1A, right panel, black arrows). Additionally, cells that did spread in gels cast with HA showed decreased levels of filopodia formation and were generally more rounded than cells seeded on gels cast without HA (Figure 1.1C, white arrows). Note that eventually all cells spread on the collagen gels regardless of the presence of HA in the microenvironment by two hours.

We next examined the dynamics of adhesion and migration for cells seeded on collagen I gels in the presence or absence of HA. Using time lapsed brightfield microscopy we monitored cells over 1.5 hours to examine the dynamics of cells migrating across these gels. When seeded on collagen gels alone, cells migrate using mainly filopodia, and cells sending out filopodia do not adhere to neighboring cells (Figure 1.1B, right panel, white arrows and supplementary movie 2). Alternatively, cells on eHA collagen matrices employed the use of lamellipodia to migrate across the gels and formed adhesions to neighboring cells (Figure 1.1B, left panel, black arrows and supplementary movie 1). Thus, matrix associated HA can alter the formation of invasive cellular phenotypes, inducing membrane ruffling and cell-cell attachments.

Previous work has indicated that CD44 interactions with HA lead to increased migration and invasion in 2D cultures, although we have demonstrated inhibition of invasion in 3D cultures [7, 12, 21, 22]. One of the main differences between 2D and 3D cultures is the context of hyaluronan. In 2D culture hyaluronan is administered soluble in the growth media while in 3D culture hyaluronan is embedded within a collagen matrix. We wanted to examine if the context of HA presentation can change cytoskeletal organization, as this dictates the types of membrane adhesions and protrusions formed by cells. To address this issue, we administered HA to collagen I gels in two different fashions. First, we cast the collagen gels with HA, embedding the HA within the gel (eHA). Next, we cast the collagen gels but added HA to the media (sHA) when cells were seeded on top of the gel. To examine eHA or sHA dependent changes in cell morphology, MDA-MB-231 (Figure 1.1C, top row) and BT-20 (Figure 1.1C, bottom row) cells were examined for the localization of filamentous actin and CD44 receptor. eHA seeded cells demonstrated lamellipodia extension of broad membrane projections in a single direction (Figure 1.1C, white arrows). Alternatively, seeding onto collagen gels without HA or with sHA leads to increased formation of stress fibers and filopodia that project in multiple directions (Fig 1c, white arrowheads). Of note, CD44 is observed localized throughout the cell membrane with increased expression in areas of membrane projections, possibly underlying its role in regulating which types of projections are formed (Figure 1.1C). These data demonstrate that eHA can inhibit the invasive phenotypes associated with mesenchymal-like cells.

HA can have distinct effects on cellular invasion based on presentation in the cellular microenvironment

In vivo, cells must move across the stroma that surrounds them before they can invade the surrounding tissue. To examine the ability of cells to cross stromal-like matrices containing HA, highly invasive MDA-MB-231 cancer cells were allowed to invade for four hours into a modified boyden chamber migration assay consisting of collagen gels embedded with increasing amounts of HA cast on 8μm pore filters. The addition of 75 μg/ml HA to the collagen gels led (eHA) to a 1.5 fold decrease in breast cancer cell invasion (P=0.001). This inhibition of invasion was found to be dose dependent from a range of 75

μg/ml to 250 μg/ml with a 1.9 fold inhibition of invasion observed at concentrations of 250 μg/ml (Figure 1.2A).

To examine whether decreased breast cancer cell invasion in the presence of HA was due to steric hindrance, we blocked the interaction between HA and its main cellular receptor, CD44, through the use of the functional CD44 blocking antibody, BRIC235. If steric hindrance was the mechanism by which HA inhibits cellular invasion, then the addition of this antibody should have no effect on levels of invasion as this does not affect the composition of HA in the gels. Preincubation of cells with BRIC235 resulted in levels of invasion similar to those seen in collagen gels lacking HA even though the cells were cast with HA ($P>0.05$)(Figure 1.2B). Note that addition of a non-specific IgG had no effect. This is indicative that the HA mediated inhibition of invasion is highly dependent on its interaction with CD44.

HA is found in nature both as part of the extracellular matrix in tissue and in a soluble form typically expressed as a pericellular coat during development [26, 27]. A number of studies demonstrate

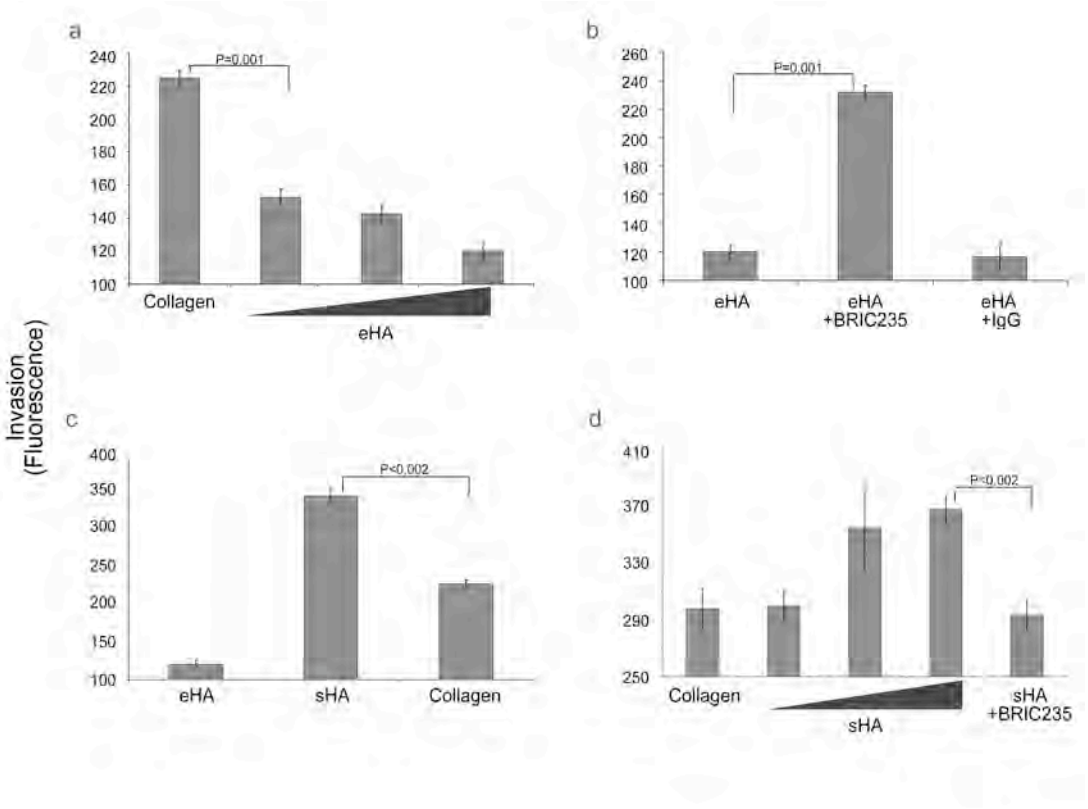

Figure 1.2 Differential HA invasive phenotypes of eHA, collagen and sHA.
(A) HA dependent inhibition of invasion is dose dependent. 10^5 cells were fluorescently tagged and placed on 1mg/ml type I collagen gels with increasing amounts of HA (75-250 µg/ml) in a modified boyden chamber invasion assay. 4 hours later relative invasion was determined by fluorescent quantification. (B) HA mediated inhibition of invasion is dependent on CD44 binding. Addition of 1µg CD44 functional blocking antibody (BRIC235) to an invasion assay as described in **a** leads to increased invasion even when on eHA gels. Addition of 1µg of rat IgG isotype control is similar to eHA alone. (C) eHA and sHA have opposite invasion phenotypes. While the addition of 125µg/ml eHA to collagen gels led to decreased invasion, the addition of 250 µg/ml sHA to collagen gels lead to a significant increase ($P<0.002$) in invasion as described in **a**. (D) sHA increases invasion at concentrations of 125 and 250 but not 75 µg/ml . Increased invasion is dependent on CD44 as demonstrated by the addition of 1µg BRIC235.

that the addition of HA to the cellular media can promote invasion [7, 21, 22]. We next set out to determine if HA affects cell invasion differently depending on if it is present soluble or as part of a matrix

in the cellular microenvironment. We observed that while eHA inhibits invasion, sHA leads to increased cell invasion when provided to cells at doses of 125 or 250 μg/ml ($P<0.002$) (Figure 1.2C and D). These effects were also CD44 dependant as the addition of the functional blocking antibody, BRIC235, led to a decrease in invasion ($P<0.01$) (Figure 1.2D). These results demonstrate that although HA embedded in a collagen matrix inhibits CD44 dependent invasion, sHA promotes CD44 dependent invasion.

Manner of HA presentation induces distinct signal transduction pathways

We have shown that cells invade less into collagen I matrices containing eHA but the molecular mechanisms mediating this inhibition are unknown. To understand how eHA can inhibit invasion, cells were seeded onto collagen I gels cast with or without eHA and protein lysates were collected after one hour. CD44 dependent invasion has been demonstrated to involve the activation of the intracellular kinases focal adhesion kinase (FAK) and Src [28]. We observed an inhibition of both FAK and Src phosphorylation in eHA versus collagen alone (Figure 1.3A). Phosphorylation of FAK at tyrosine 397 as well as Src phosphorylation at tyrosine 416 (both activating modifications that induce cell invasion) were decreased in cells seeded onto collagen I gels cast with HA [29]. The addition of a CD44 functional blocking antibody, BRIC235, partially rescued both FAK and Src phosphorylation indicating that CD44 is involved in this signaling.

The activation of Src and FAK can lead to the induction of a number of signal transduction pathways that can affect cellular invasion [30, 31]. Two of these downstream effectors are p21-activated

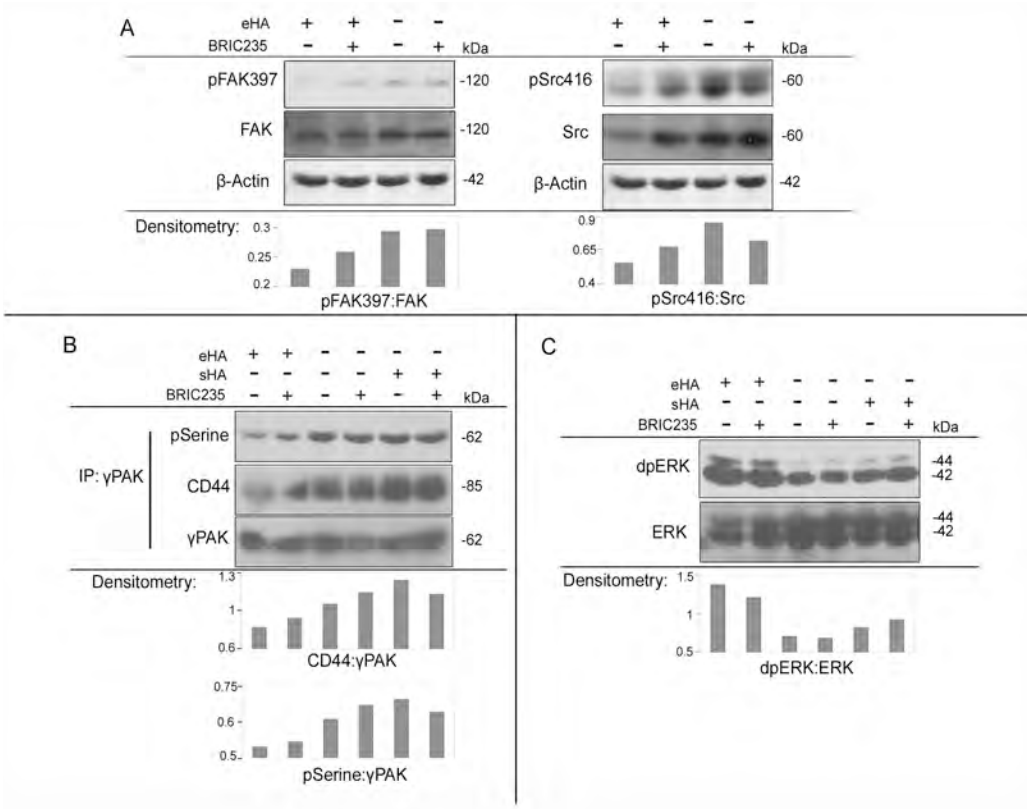

Figure 1.3 Intracellular signaling is altered by eHA and sHA treatments.
(A) Lyastes from $2x10^6$ cells placed on 1mg/ml type I collagen gels with or without 125µg/ml eHA and sHA were run on SDS-PAGE electrophoreses gels and immunoblotted. FAK397 and Src416 phosphorylation is decreased in cells placed on eHA gels but not collagen gels or sHA gels. Relative expression densitometries are indicated. (B) Lysates of cells seeded on eHA, collagen or sHA gels as described in A**,** were subject to immunoprecipitation and immunoblotting. eHA gels lead to decreased CD44 association with γPAK and decreased γPAK p-serine. Relative densitometries are indicated. (C) Lysates of cells as described in B were subject to immunoblotting for dual phosphorylated (dp) ERK as well as pan ERK. Relative densitometries shown below.

kinase (PAK) and ERK. PAK is known to alter invasion and metastasis by modulating the actin cytoskeleton through the activation of LIMK and ARP2/3 [32-34]. Analyzing protein-protein interactions from cells seeded on collagen alone or collagen +eHA or sHA, we observed a decrease in CD44 interactions with γPAK in the presence of eHA which was dependent on CD44 (Figure 1.3B, top panel). Alternatively, we observed an increase in CD44 interactions with γPAK in sHA gels which was not

blocked by CD44 blocking antibodies (Figure 1.3B, top panel). The levels of binding of CD44 to γPAK closely correlate with γPAK activation as demonstrated by serine phosphorylation of γPAK. We found that γPAK is highly phosphorylated in sHA contextbut phosphorylation is decreased in the presence of eHA (Figure 1.3B, middle panel).

We next examined how HA affects ERK activation. In contrast to the decreased activation observed with γPAK, ERK kinase is activated when cells encounter eHA (Figure 1.3C, top panel). This increase is not observed in the presence of sHA. However, while ERK activation is clearly dependant on eHA, it was not dependant on CD44 as demonstrated by the addition of BRIC235 (Figure 1.3C, top panel). Together, these data demonstrate an eHA-dependent activation of ERK and suppression of FAK, Src and γPAK activation. Alternatively, sHA appears not to regulate ERK while it promotes γPAK activation.

PAK and ERK differentially regulate HA dependent invasion

We next evaluated the reliance of HA-dependent invasion on PAK and ERK activity. For these studies we utilized the PAK inhibitor PAK18, or the ERK activation inhibitory peptide, in our invasion assays [35]. When invading into collagen gels that do not contain any HA, the addition of PAK18 led to a significant decrease in invasion (P=0.003) (Figure 1.4A). As demonstrated previously, the addition of BRIC235 does not affect invasion of breast cancer cells into collagen gels with no HA. Similarly the simultaneous addition of PAK18 and BRIC235 gave no additional decrease beyond PAK18 alone (Figure 1.4A). This demonstrates that when invading into collagen gels in the absence of HA, cells do not rely on CD44 for invasion but do rely on PAK activation.

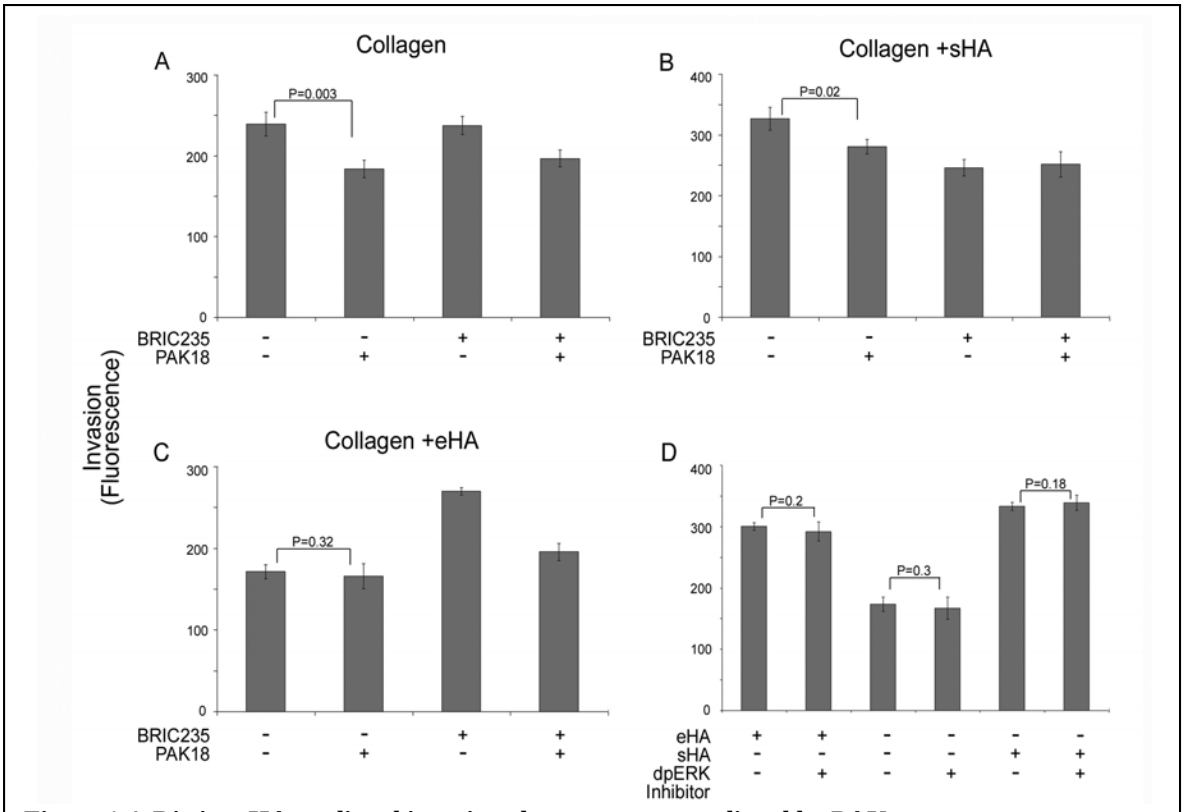

Figure 1.4 Distinct HA mediated invasive phenotypes are mediated by PAK.
10^5 Cells were subject to invasion assays as decribed in Fig. 2 A. CD44 functional blocking (BRIC235), 35μM PAK18 or 10μM dpERK inhibitor were added where indicated. Cellular invasion is mediated by PAK activity in collagen (A) and sHA gels (B), but not in eHA gels (C). Invasion was not dependent on ERK activity in any of the conditions (D).

We next examined the role of PAK during sHA mediated invasion. Treatment of cells with PAK18 led to a significant decrease in invasion (P=0.02), similar to the decrease observed with CD44 blocking antibody (Figure 1.4B). Examination of cells invading in eHA demonstrated no PAK mediated effects (P=0.32) (Figure 1.4C).

Using an ERK activation inhibitory peptide, we next investigated the contribution of ERK activation to cell invasion into collagen gels [36]. ERK has previously been reported to promote invasion by enhancing the activity of myosin light chain kinase [37]. Inhibition of ERK activation did not significantly affect cell invasion into collagen gels under any conditions, including collagen alone, eHA or sHA (Figure 1.4D). These results indicate that although ERK is activated by eHA (Figure 1.3C), it plays no role in invasion.

Soluble or matrix bound HA can have diverse proliferative phenotypes.

We next evaluated whether HA dependent changes in invasion were related to changes in cell number. We seeded cells on collagen gels cast with and without HA and allowed them to proliferate. Cells were extracted from the gels two and three days after seeding and counted by trypan blue exclusion (Figure 1.5A). We observed a significant increase in cell number in cells grown in eHA after three days ($P<0.008$)(Figure 1.5A). These data suggest a possible role for HA in regulating cell proliferation in addition to its role in regulating invasion.

To evaluate the role of eHA and sHA in proliferation, we perfomed an MTT assay. Cells were seeded on collagen gels in the presence of either eHA or sHA and allowed to grow for five days, after which time, cell numbers were determined with an MTT assay (Figure 1.5B). As with cellular invasion, the addition sHA had the opposite effect to that seen with eHA on cells. While cells showed significantly elevated levels of proliferation when grown in the presence of eHA compared to collagen alone (P<0.001),

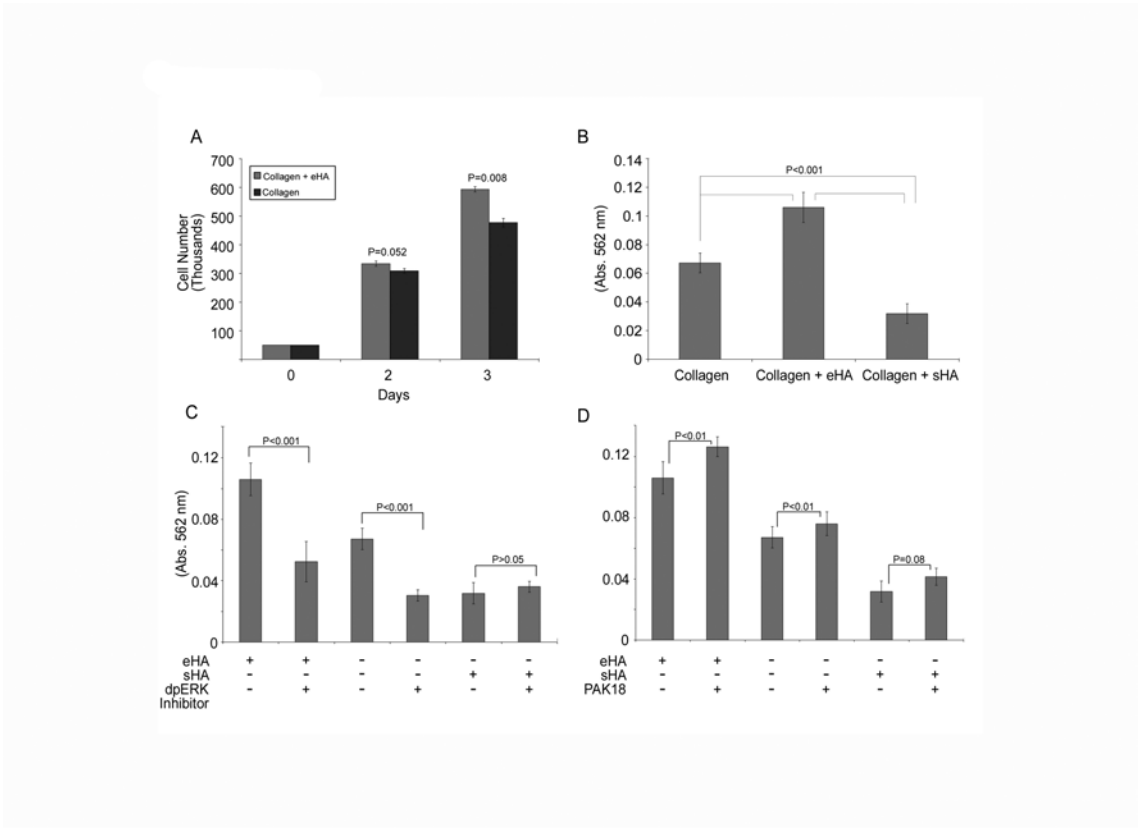

Figure 1.5 Differential HA proliferative phenotypes of eHA, collagen and sHA.
(A) 10^4 cells were seeded on 1mg/ml type I collagen gels with or without 125µg of eHA or sHA. Cell placed on eHA gels demonstrated significant increased (P=0.008) cell numbers compared to collagen gels alone, 3 days after seeding, as enumerated by trypan blue exclusion. (B) Quantification of cell number by MTT assay shows distinct proliferative phenotypes of 125 µg/ml eHA and sHA gels. While eHA gels lead to a significant increase in cell numbers (P<0.001), sHA gels lead to significantly decreased cell numbers (P<0.001) 5 days after seeding $5x10^3$ on collagen gels. (C) Increased cell numbers are dependent on ERK activity in eHA and collagen gels but not in sHA gels as demonstrated by the addition of 10µM ERK activation inhibitor. (D) Inhibition of PAK by 35µM PAK18 leads to increased cell numbers in eHA and collagen gels but not in sHA gels.

reduced proliferation was observed when cells were grown in sHA compared to collagen alone (P<0.001)

(Figure 1.3C). To rule out the possibility that HA mediated decreases in cell number were due to apoptosis, we performed an Annexin V/Propidium iodide apoptosis assay in gels cast with or without HA. No significant differences were observed (P=0.072) whether or not HA was present in the gel indicating that increases in proliferation are not due to increased apoptosis (Data not shown).

Given the ability of ERK to mediate cell growth and the fact that it is activated in the presence of eHA (Figure 1.3C), we next examined the role of ERK activation HA mediated proliferation. Cells grown in the presence or absence of the ERK activation inhibitor were grown for five days on collagen, eHA collagen or sHA collagen and evaluated using an MTT assay (Figure 1.5C). Inhibition of ERK led to significant decreases in cell number when cells were seeded onto eHA or collagen alone ($P<0.001$) (Figure 1.5C). However, no significant effect was seen in cells seeded on sHA collagen (Figure 1.5C).

Since eHA and sHA have displayed opposite invasion and proliferation phenotypes and have differential effects on ERK for proliferation, we next wanted to determine if they differentially utilized PAK for proliferation. Inhibition of PAK in cells seeded onto collagen or eHA collagen led to a significant increase in cell proliferation ($P<0.01$) (Figure 1.5D). This indicates that while PAK activation is promoting invasion (Figure 1.4), PAK is also inhibiting proliferation (Figure 1.5D). As with the ERK inhibitor, the addition of PAK18 to cells seeded on collagen gels with sHA did not lead to any significant changes in cell proliferation (P=0.08). These data demonstrate that HA embedded in a collagen matrix inhibits invasion while promoting proliferation in an ERK and PAK dependent manner. Alternatively, sHA promotes invasion in a PAK dependent manner, but does not significantly impact proliferation via either ERK or PAK.

In this study, we identify HA as a component of the extracellular matrix that differentially alters invasion and proliferation depending on its presentation in the cellular microenvironment. In the presence of sHA, cells efficiently invade into collagen gels while proliferation is inhibited. Conversely, when interacting with eHA, cells decrease spreading and invasion into the collagen gels while increasing proliferation. When HA is absent from the cellular microenvironment, cells adhere, invade and proliferate at rates in between those seen with soluble or matrix associated HA. The differential effects seen in the presence of sHA and eHA are mediated by ERK and PAK, as demonstrated through the use of their inhibitors in

invasion and proliferation assays, and analysis of protein expression and activation. Overall, these results demonstrate that the context in which HA is presented to the cells alters signaling, invasion and proliferation. Furthermore, cells are restricted to either 'go' or 'grow' phenotypes indicating that the microenvironment context can strongly regulate cellular phenotypes.

The mechanisms employed by cells to migrate across collagen I matrices are modulated by HA and dependant on its presentation. Cells mainly employ the use of lamellipodia for migration and make strong adhesions to neighboring cells when seeded on collagen gels cast with HA. However, cells use mainly filopodia when migrating and move away from neighboring cells when seeded on collagen I gels lacking any HA. This might underscore the mechanisms by which CD44/HA interactions may inhibit metastasis as previously seen in mouse models of breast cancer [12]. The formation of strong attachments to neighboring cells may impede tumor cells from migrating away from its primary site when HA is present in the desmoplasia, providing an additional obstacle against metastasis. Alternatively, sHA production by tumor epithelial cells becomes localized to the glycocalyx around the cells that may serve to isolate them from matrix components.

The ability of cancer cells to invade requires the use of a number of cellular resources, such as the cytoskeleton, that are shared by other cellular processes such as cellular proliferation. In fact, it has been proposed that cells cannot engage in both invasion and proliferation simultaneously as they both are heavily reliant similar resources and this has led to the formation of the "Go or grow" hypothesis [38]. The dramatic inhibition of invasion across collagen matrices containing eHA correlated with a strong increase in proliferation of cells. This change to a proliferative phenotype appears to be both ERK and PAK dependent. While proliferation is promoted by ERK (as demonstrated by a reduction of proliferation in the presence of an ERK inhibitor), it is similarly inhibited by PAK (as demonstrated by an increase in proliferation in the presence of a PAK inhibitor). These findings lend support to the idea that cells must allocate resources to accommodate for increased invasion or proliferation, but cannot engage fully in both simultaneously.

PAK activation and association with CD44 is dependent upon HA/CD44 interactions as demonstrated by BRIC235 treatment. Alternatively, BRIC235 treatment demonstrated that ERK activation, while increased in the presence of eHA, is not dependent upon HA/CD44 interactions. This

may indicate that in some circumstances eHA may be depending on alternate HA receptors for ERK mediated effects. Receptor for HA mediated motility (RHAMM) is a cell surface receptor that binds HA and has been recently shown to mediate ERK signaling to affect proliferation [39-41]. Therefore, it will be interesting to determine if eHA induced ERK activation is RHAMM dependent in future studies.

Our finding that HA presentation alters cellular invasive and proliferative phenotypes sheds light on conflicting results for the role of HA in tumor progression as HA can both inhibit and promote proliferation and invasion (Figure 1.6, model). When cells encounter eHA, there is decreased binding of CD44 to PAK that corresponds with a lack of PAK serine phosphorylation and activation. Despite decreased PAK activity, we demonstrated a PAK mediated inhibition of proliferation even in the presence of eHA. Nonetheless, cells grown on eHA maintain elevated levels of proliferation as a result of CD44 independent ERK activation. In contrast to this, when cells encounter sHA there is increased CD44 association with PAK and a corresponding increase in PAK serine phosphorylation and activation that ultimately leads to increased invasion. While sHA leads to greater cell invasiveness, it also causes cells to slow down proliferation in a PAK or ERK independent manner

Maintenance of proper interactions between the epithelium and the stroma are important for the preservation of normal tissue architecture that keeps tumor cells confined to their primary sites. While studying HA as a soluble component of the cellular microenvironment has important *in vivo* correlates, such as during wound healing and development [6, 42, 43], epithelial cells encounter fibroblast-deposited HA mainly as part of the extracellular matrix [44]. In addition, HA synthase expression in epithelial cells can produce soluble HA that promotes metastatic invasion of cells freed from the tissue microenvironment, such as cells in circulation [45]. Cells have a wide range of behaviors that they can adopt after binding matrix components, but it is becoming increasingly clear that cells sense not only the presence of ligands in the microenvironment, but also the way in which ligands are presented and the forces generated behind them. In this study we demonstrate that HA can have widely different effects on cells depending on whether it is soluble or part of a matrix in the cell microenvironment, and underscores the importance of studying matrix components in a manner that closely resembles what cells encounter in nature.

SECTION II. CD44/HYALURONAN INTERACTIONS REMODEL THE TUMOR MICROENVIRONMENT TO INHIBIT METASTASIS

The acquisition of motile phenotypes is an essential step in achieving invasion and metastasis, the deadliest of the hallmarks of cancer. The connective tissue framework supporting organs, referred to as the stroma, and immune cell surveillance comprise major obstacles for metastasizing cells. The only stromal element that non-transformed epithelial cells typically encounter during their lifetime is the basal lamina, separating the epithelial cell from the adjacent interstitial tissue. The confinement of epithelial cells in the

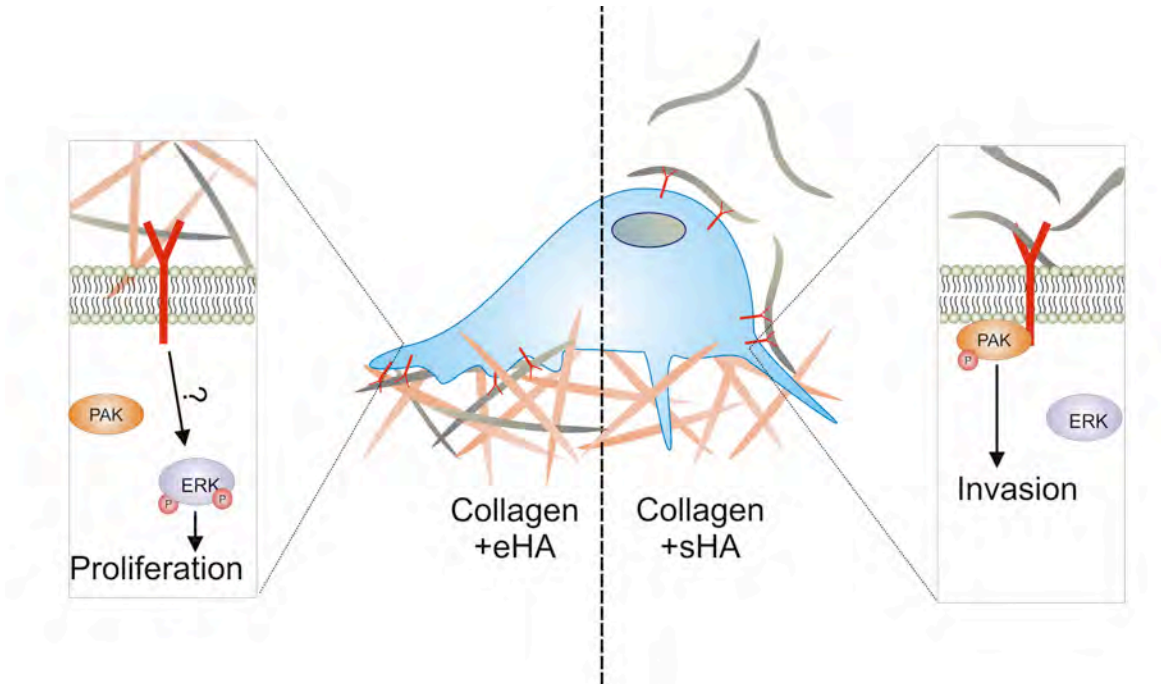

Figure 1.6 Model of differential effects of byaluronan based on presentation in the microenvironment. When binding to eHA, cells migrate mainly through the use of lamellipodia as invasion is inhibited. Interactions between the surface receptor CD44 and PAK as well as PAK activation are decreased as CD44 binds eHA. Even with low levels of active PAK, there still exists a PAK mediated inhibition of proliferation. Cells on eHA maintain elevated levels of proliferation, however, through CD44 independent ERK activation. Alternatively, when cells bind collagen gels this HA soluble in the microenvironment, cells invade mainly through the use of filopodia. As CD44 binds sHA, there is an increased association between CD44 and PAK that leads to increased PAK activity and invasion. Interestingly, while sHA increases invasion, it also inhibits proliferation.

mammary gland to ductal structures normally occurs via interactions between the epithelium and neighboring cells called adherens junctions, as well as through interactions between the epithelial cell and

the basal lamina, such as hemidesmosomes [46, 47]. Additional steric barriers to metastasis exist in the stroma in the form of connective tissue, which is comprised heavily of collagen I, that normally also inhibit cellular movement through tissues. To successfully move through the stroma, tumor cells must gain the ability to degrade the various components of the extracellular matrix, typically by secreting proteases to open spaces through which the tumor can travel. However, while traveling through the stroma tumor cells can also encounter resident macrophages. Sentinel macrophages help maintain tissue homeostasis by eliminating pathogens or damaged cells, including transformed cells, via induced apoptosis. Immune cells dock onto transformed or damaged cells, and release a combination of apoptosis inducing factors such as Fas, TNF or TRAIL, as well as perforin, a protein which causes membrane rupturing [48]. Tumor cells, overcome obstacles imposed by the stroma and immune system by secreting cytokines that inhibit immune response, such as TGF-β, through the devleopment of immune tolerace, by remodelling the extracellular matrix or by highjacking the immune system to aid it during migration and extravasation.

Matrix metalloproteases (MMP) are a family of greater than 20 endopeptidases that can collectively cleave most components of the extracellular matrix [4]. A number of different MMPs are secreted by tumors or tumor-associated cells, such as fibroblasts and immune cells, which function mainly to degrade the extracellular matrix so that tumor cells are not limited by interactions with components of the extracellular matrix as they move through the stroma (Figure 2.1). A large amount of evidence collected from mouse models of cancer indicate that MMPs are an essential component in the acquisition of invasive phenotypes in a number of cancers including breast cancer. MMP1 and MMP7 overexpression in mice leads to hyperplasia while overexpression of MMP3 or MMP14 was sufficient to induce spontaeous breast cancers in mice [49-51]. MMP2, 7, 9 and 11 knowckout mice developed fewer cancers than wildtype mice underscoring the role of some MMPs tumorigenesis [49]. Additionally, tumor cells injected into MMP9 null mice failed to invade and colonize the lungs whereas those injected into wildtype mice colonized the lungs successfully. These data indicate an important role for MMP9 and other MMPs in the establishment of distant tumor colonies [52].

Tumor cells further remodel the extracellular matrix by recruiting stromally resident cells, such as fibroblasts and macrophages [5, 10, 11, 53-57]. A dynamic interaction exists between breast tumor cells

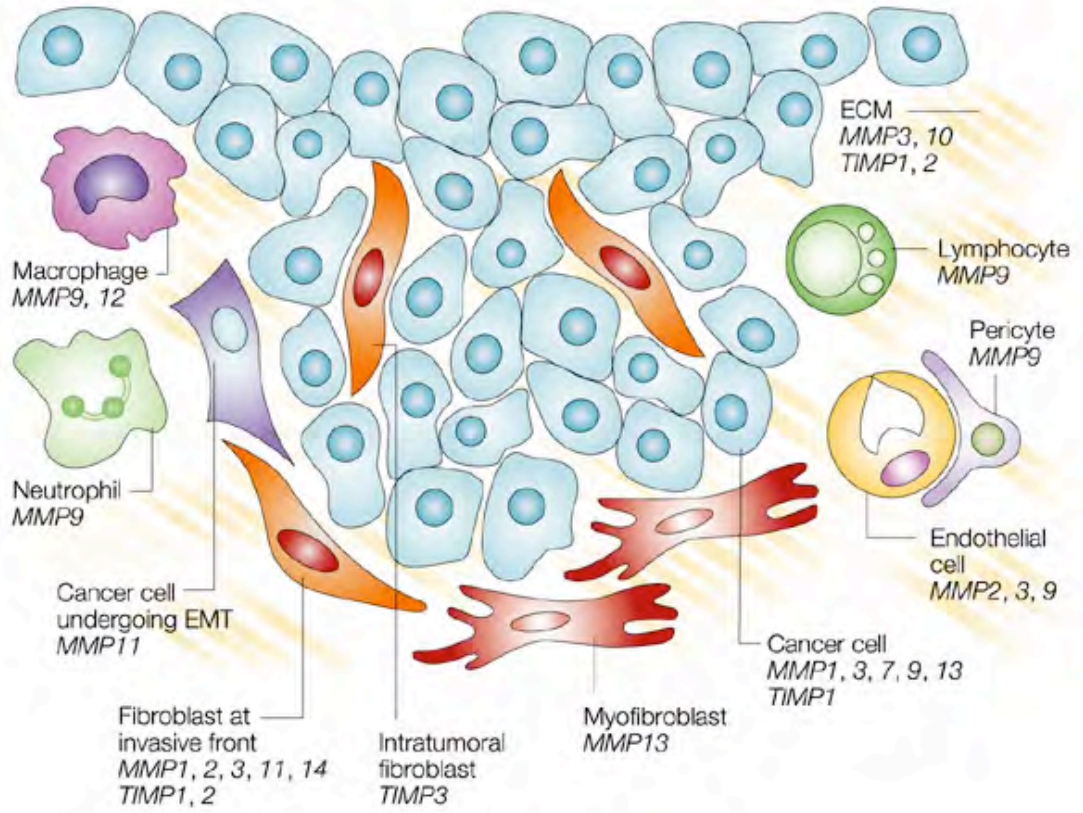

Figure 2.1: MMPs in Mammary Cancers:
During tumorigenesis, a variety of cell types produce MMPs responsible for degrading extracellular matrix components that can facilitate tumor cell movement. Cells resident in the stroma, such as fibroblast and macrophages, are induced to produce MMPs by tumor cells. This figure was adapted from [4]

and macrophages that enable cell movement away from the primary site. In fact, elimination of the macrophage population from a metastatic mouse model of cancer (PyV mT model), led to an almost complete ablation of metastasis [54]. Through the use of the PyV mT mouse model, it was demonstrated that tumor cells recruit macrophages by secreting colony stimulating factor 1 (CSF-1), a step essential for their metastatic ability. Secretion of MMPs by macrophages can lead to the proteolytic release of hypoxia-induced growth factors, such as vascular endothelial growth factor (VEGF), that increase angiogenesis and vascularization of the tumor. In fact, mouse mammary cancer cells have been observed using intravital

confocal microscopy, migrating with tumor resident macrophages through mammary tissue and intravasate into the vasculature together [55].

Previous research conducted in our lab has indicated a strong repression of breast cancer invasion when cells are placed on eHA containing matrices [12]. These observations were made utilizing mouse models of metastatic breast cancer, and recapitulated *in vivo*, utilizing 3D collagen I invasion assays with eHA. While a lot of research is focused on how CD44 interactions with HA affect tumor cells themselves, few studies have examined how these interactions may facilitate tumor cell remodeling of the tumor-associated stroma, or how they may affect immune function as it pertains to metastasis. Understanding this becomes relevant in light of recent studies indicating that the interaction between CD44 and HA regulates cellular MMP9 transcriptional activation. In osteoclast-like cells, the interaction between CD44 and HA was shown to strongly down-regulate MMP9 transcription, which inhibited cellular migration [58]. If HA dependent MMP9 regulation is conserved in tumor epithelial cells, then this may comprise a key mechanism utilized by the desmoplasm and HA to inhibit cellular movement.

In this study, we show evidence indicating that stromal HA interactions with CD44 can inhibit MMP9 mediated invasion. The inhibition of MMP9 depends on the context in which HA is presented. If HA is present embedded within a collagen I matrix, no effect is seen on MMP9 mediated invasion. Alternatively, if HA is present embedded in a collagen IV matrix, a strong inhibition of MMP9 mediated invasion is observed. We also present *in vivo* evidence indicating the absence of pulmonary macrophages in CD44 null mice. While macrophages have been implicated in tumor metastasis, their pulmonary sentinel activities may be important for preventing the growth of metastatic breast tumors in the lungs.

Results

CD44 interactions with HA does not suppress the transcriptional activity of MMP9 when HA is embedded within a collagen I matrix.

Our previous findings indicating that HA can inhibit breast tumor cell invasion when in a collagen matrix show that HA does not inhibit tumor cell invasion through steric hindrance [12]. The incubation of breast cancer cells with a functional blocking antibody designed to inhibit the interaction between HA and CD44 led to high levels of invasion even while HA was present in the media. This indicates that HA inhibits invasion by mechanisms involving its primary receptor, CD44. Bearing in mind recent findings

indicating that CD44 interactions with HA can decrease MMP9 expression by inhibiting its transcriptional repression, we sought to investigate whether CD44 dependent regulation of MMP9 occurs in breast cancer cell lines [58]. CD44 transcriptional inhibition of MMP9 in breast cancer cells is highly plausible especially considering previous studies indicating that in breast cancer cells, cleavage of CD44 can lead to the nuclear translocation of the CD44 cytoplasmic tail where it regulates the activity of TPA-response elements (TRE) [59]. The MMP9 promoter region contains a TRE element making it a possible target for transcriptional regulation by CD44 [60].

To investigate whether CD44 interactions with HA could promote the transcriptional activation or repression of MMP9, we cotransfected the metastatic breast cancer cell line, MDA-MB-231 with 2 expression plasmids that would enable us to accurately measure MMP9 transcriptional activity while correcting for transfection efficiency. The first construct used was a plasmid that expresses the luciferase gene under the control of the MMP9 promoter (MMP9-luc). We cotransfected this construct with a second plasmid that constitutively expresses secreted alkaline-phosphatase (SEAP) under the CMV promoter. We utilized the constitutive SEAP construct in tandem to the MMP9-luc construct as a baseline to measure our transfection efficiency to effectively rule out the possibility that differences in MMP9 promoter activity might be due to different levels of plasmids within the cells.

After cotransfection of the MDA-MB-231 cells with the plasmid, cells were seeded onto type I collagen gels cast with or with HA and incubated for 24 hrs. The growth media was then collected and cells were lysed to measure SEAP secretion and luciferase expression in the cells. No significant differences in MMP9 promoter activity, as measured by luciferase expression, were observed with or without HA in the collagen gel (Fig 2.2). Additionally, treatment of cells with either the CD44 functional blocking antibody, BRIC235, or an MMP2/9 inhibitor does not significantly ($P>0.05$) affect MMP9 promoter readout. TPA was included as a positive control as it has previously been shown to induce MMP9 promoter activity and MMP9 gene transcription. While there was a slight increase in promoter activity, it was not significantly higher than the untreated controls, indicating that MDA-MB-231 cells have high constitutive expression levels of the MMP9 gene [60].

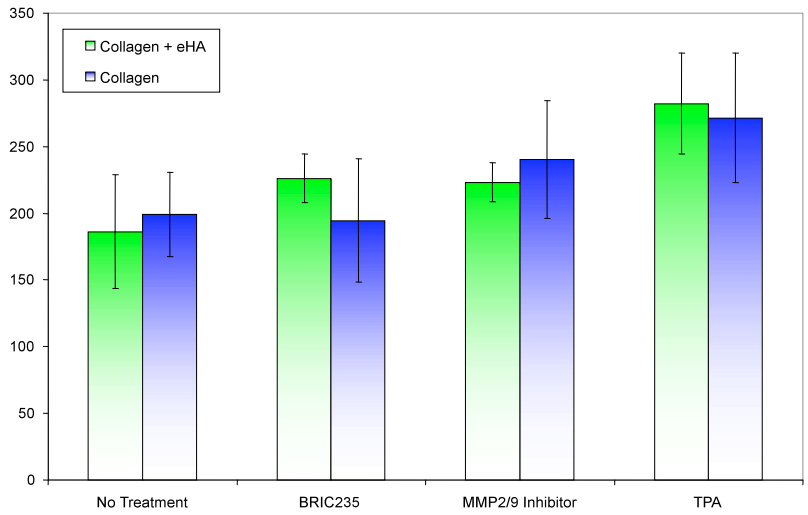

Figure 2.2: CD44 interactions with HA do not alter the transcriptional activity of the MMP9 promoter on type I collagen

10^6 cells seeded onto collagen I gels were cotransfected with the MMP9-luc and CMV-SEAP constructs to assay for MMP9 promoter activity. Cells were lysed and assayed for luciferase activity. The addition of HA did not change MMP9 prmoter activity. Blocking of CD44 by BRIC235 addition, or the addition of MMP2/9 inhibitors did not alter MMP9 levels. The addition of TPA cells did increase MMP9 promoter activity as it contains a TRE element controlling its transcriptional activation.

Hyaluronan interactions with CD44 do not affect MMP2/9 dependent invasion into collagen I matrices

Transcriptional regulation of the MMP9 gene is just one of the many different levels of regulation that MMP9 is subject to. Post-translational modifications, proper secretion of MMPs into the extracellular space, or cleavage of the pro-form of the MMP to yield an active MMP in the extracellular space are also required for MMP9 protein activity. It

remains plausible that CD44 interactions with HA may be influencing MMP9 activity at a different level of regulation. Since it has been previously demonstrated that MMP activity heavily affects breast cancer cell invasion phenotypes, we were interested in examining whether CD44/HA interactions might affect MMP2/9 dependent invasion through the use of a 3D invasion assay utilizing and MMP2/9 specific inhibitor.

Collagen I gels cast with or without HA were poured below transwell inserts containing 8μm pores. The gels were re-hydrated in cellular growth media containing 20% FBS while cells were seeded in the upper portion of the chamber in serum free media. The FBS gradient across the 8μm pores serves as a chemotactic gradient that draws cells from the upper chamber into the gel in the bottom chamber of the invasion assay.

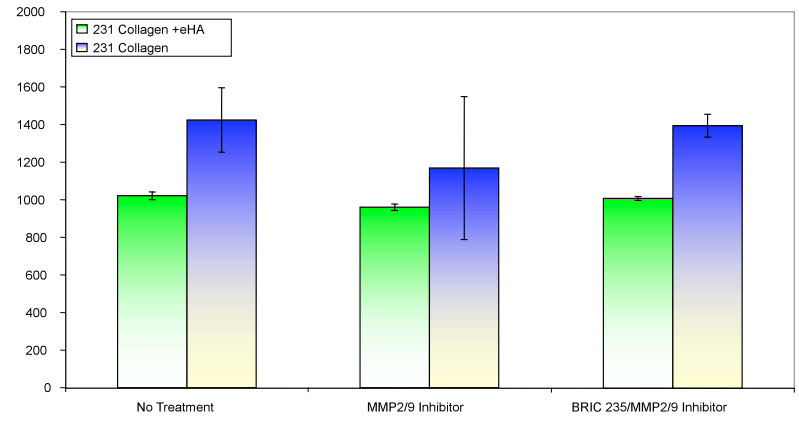

Figure 2.3: MMP2/9 Dependent Invasion into type I collagen is not modulated by matrix HA. Invasion assays were performed as described in Figure 3.4. eHA did not alter the MMP9 dependent invasive phenotype as the addition of an MM2/9 inhibitor did not alter cellular invasion. Addition of BRIC235 along with the MMP2/9 inhibit similarly did not alter invasion both in the presence or absence of eHA. This indicates invasion into collagen I gels by MDA-MB-231 cells does not depend on MMP2/9 proteolytic activity in the context of eHA.

As previously described, gels cast with HA showed decreased invasion into collagen I as compared to collagen I gels without HA. However, the addition of an MMP2/9 inhibitor did not alter the levels of invasion in either the collagen I gels alone, or in the collagen I gels with HA. To assess if CD44 activity might be responsible for MMP2/9 dependent invasion, we added the CD44 blocking antibody, BRIC 235, along with the MMP2/9 inhibitor. As seen previously, addition of the CD44 blocking antibody to collagen gels lacking HA did not alter invasion, even in the presence of the MMP2/9 inhibitor (Figure 2.3). This indicates that invasion into collagen I gels, in the absence of HA, does not rely on MMP2/9 activity. The

addition of BRIC 235 along with the MMP2/9 inhibitor in collagen I gels with HA, also demonstrated no differences in invasion.

CD44 interactions with HA lead to decreased MMP2/9 dependent invasion into collagen IV gels.

Previous data have shown that the cellular phenotypes are differentially affected by HA based on how HA is present in the microenvironment (Figure 1.1). Here, we have examined the effects of HA present in collagen I matrices on MMP9 activity. Collagen I is a fibrilar collagen that forms 3D matrices and is found in the stroma throughout the body. However, the basal lamina remains the primary barrier that maintains epithelial cell localization and this is composed mainly of type IV collagen. Tissue staining of mouse mammary glands indicates that while HA is highly expressed in the stroma surrounding tumors, but it remains difficult to examine the tumor basement membrane in the presence of desmoplasm due to the increased deposition of matrix components (Figure 3.2D). Instead, we examined the normal mammary gland for HA expression, as the basement membrane is much easier to visualize in the absence of a reactive stroma (Figure 3.2C, arrowhead). In the normal mammary gland, HA is present in the basement membrane surrounding the ductal epithelial structures with very little HA expression in the surrounding stroma, indicating a strong association of HA in the collagen IV compartment of the extracellular matrix (Fig 3.2 C and D).

In view of the presence of HA in the basement membrane surrounding mammary ducts, and the importance of collagen IV and the basal lamina in the maintenance of normal tissue architecture and homeostasis, we wanted to examine how CD44 interactions with HA in a collagen IV environment can affect MMP2/9 proteolytic activity and invasion. Understanding this also becomes highly relevant as we have previously demonstrated that HA can mediate phenotypes differentially depending in the microenvironment. We were interested to see if HA mediated activities depended not only on its integration into a matrix, but also if the matrix itself could alter HA phenotypes.

We performed and invasion assay as described above, with collagen IV matrices instead of collagen I gels, with or without HA on 8μm pore filters. We established a similar chemotactic gradient as described above by placing cellular growth media with 20% FBS on the bottom chamber while cells were seeded onto the top chamber of the transwell in serum free media. Cells were allowed to invade for 24 hrs before being enumerated.

The presence of HA in a collagen IV matrix inhibited cellular invasion in a manner similar to that observed in collagen I matrices. The addition of a CD44 blocking antibody, BRIC 235, increased invasion in collagen IV gels containing HA. This indicates that HA inhibits invasion by a mechanism that is dependent on its binding to CD44. The administration of an MMP2/9 inhibitor to the cells while invading did not lead to any changes in invasion both in the collagen IV gels alone, or the collagen IV matrix containing HA. However, when both BRIC 235 and the MMP2/9 inhibitor were added simultaneously, invasion into collagen IV matrices with HA remained at levels similar to those seen with either the MM2/9 inhibitor alone, or without the addition of any factors. This indicates that in the absence of CD44 binding, high levels of invasion into collagen IV matrices containing HA depends on MMP2/9 activity. This activity, however, is inhibited when HA binds CD44.

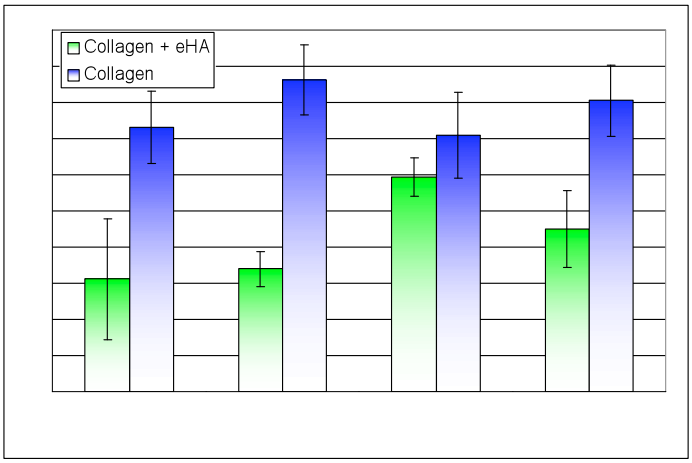

Figure 2.4: MMP2/9 dependent inhibition of invasion occurs in the presence of eHA while in a collagen IV matrix. Cells were seeded onto an invasion assay as in Figure 5.3 but instead of a collagen I matrix, eHA was embedded within a collagen IV matrix. The addition of BRIC235 into collagen IV matrices with eHA increases cellular invasion that is abrogated with the addition of an MMP2/9 inhibitor. This indicates that cells rely on MMP2/9 proteolytic activity to invade through Collagen IV in the presence of eHA.

Key Research Accomplishments:

- Identified HA as a component of the extracellular matrix that differentially alters invasion and proliferation depending on microenvironmental presentation.
- In the presence of soluble HA cells invade while proliferating at low rates in and ERK and PAK mediated mechanism.
- In the presence of embedded HA, cells proliferate highly while displaying low invasiveness.
- CD44 interactions with HA do not affect MMP9 transcription of breast tumor cells seeded on collagen I gels.
- In the absence of HA binding, breast tumor cells increase MMP9 transcription when seeded on Collagen IV gels.

Reportable Outcomes:

Degrees: Completed Ph.D. in Molecular and Celluar Biology at the University of Arizona
Employment/Research Opportunities: Currently employed in the Department of Surgery at the University of California, San Francisco as a Postdoctoral Research Fellow.

Conclusions.

In this study, we identify HA as a component of the extracellular matrix that differentially alters invasion and proliferation depending on its presentation in the cellular microenvironment. In the presence of sHA, cells efficiently invade into collagen gels while proliferation is inhibited. Conversely, when interacting with eHA, cells decrease spreading and invasion into the collagen gels while increasing proliferation. When HA is absent from the cellular microenvironment, cells adhere, invade and proliferate at rates in between those seen with soluble or matrix associated HA. The differential effects seen in the presence of sHA and eHA are mediated by ERK and PAK, as demonstrated through the use of their inhibitors in invasion and proliferation assays, and analysis of protein expression and activation. Overall, these results demonstrate that the context in which HA is presented to the cells alters signaling, invasion and proliferation. Furthermore, cells are restricted to either 'go' or 'grow' phenotypes indicating that the microenvironment context can strongly regulate cellular phenotypes.

The mechanisms employed by cells to migrate across collagen I matrices are modulated by HA and dependant on its presentation. Cells mainly employ the use of lamellipodia for migration and make strong adhesions to neighboring cells when seeded on collagen gels cast with HA. However, cells use mainly filopodia when migrating and move away from neighboring cells when seeded on collagen I gels lacking any HA. This might underscore the mechanisms by which CD44/HA interactions may inhibit metastasis as previously seen in mouse models of breast cancer [12]. The formation of strong attachments to neighboring cells may impede tumor cells from migrating away from its primary site when HA is present in the desmoplasia, providing an additional obstacle against metastasis. Alternatively, sHA production by tumor epithelial cells becomes localized to the glycocalyx around the cells that may serve to isolate them from matrix components.

The ability of cancer cells to invade requires the use of a number of cellular resources, such as the cytoskeleton, that are shared by other cellular processes such as cellular proliferation. In fact, it has been proposed that cells cannot engage in both invasion and proliferation simultaneously as they both are heavily reliant similar resources and this has led to the formation of the "Go or grow" hypothesis [38]. The dramatic inhibition of invasion across collagen matrices containing eHA correlated with a strong increase

in proliferation of cells. This change to a proliferative phenotype appears to be both ERK and PAK dependent. While proliferation is promoted by ERK (as demonstrated by a reduction of proliferation in the presence of an ERK inhibitor), it is similarly inhibited by PAK (as demonstrated by an increase in proliferation in the presence of a PAK inhibitor). These findings lend support to the idea that cells must allocate resources to accommodate for increased invasion or proliferation, but cannot engage fully in both simultaneously.

PAK activation and association with CD44 is dependent upon HA/CD44 interactions as demonstrated by BRIC235 treatment. Alternatively, BRIC235 treatment demonstrated that ERK activation, while increased in the presence of eHA, is not dependent upon HA/CD44 interactions. This may indicate that in some circumstances eHA may be depending on alternate HA receptors for ERK mediated effects. Receptor for HA mediated motility (RHAMM) is a cell surface receptor that binds HA and has been recently shown to mediate ERK signaling to affect proliferation [39-41]. Therefore, it will be interesting to determine if eHA induced ERK activation is RHAMM dependent in future studies.

Our finding that HA presentation alters cellular invasive and proliferative phenotypes sheds light on conflicting results for the role of HA in tumor progression as HA can both inhibit and promote proliferation and invasion (Figure 1.6, model). When cells encounter eHA, there is decreased binding of CD44 to PAK that corresponds with a lack of PAK serine phosphorylation and activation. Despite decreased PAK activity, we demonstrated a PAK mediated inhibition of proliferation even in the presence of eHA. Nonetheless, cells grown on eHA maintain elevated levels of proliferation as a result of CD44 independent ERK activation. In contrast to this, when cells encounter sHA there is increased CD44 association with PAK and a corresponding increase in PAK serine phosphorylation and activation that ultimately leads to increased invasion. While sHA leads to greater cell invasiveness, it also causes cells to slow down proliferation in a PAK or ERK independent manner

Maintenance of proper interactions between the epithelium and the stroma are important for the preservation of normal tissue architecture that keeps tumor cells confined to their primary sites. While studying HA as a soluble component of the cellular microenvironment has important *in vivo* correlates, such as during wound healing and development [6, 42, 43], epithelial cells encounter fibroblast-deposited HA mainly as part of the extracellular matrix [44]. In addition, HA synthase expression in epithelial cells

can produce soluble HA that promotes metastatic invasion of cells freed from the tissue microenvironment, such as cells in circulation [45]. Cells have a wide range of behaviors that they can adopt after binding matrix components, but it is becoming increasingly clear that cells sense not only the presence of ligands in the microenvironment, but also the way in which ligands are presented and the forces generated behind them. In this study we demonstrate that HA can have widely different effects on cells depending on whether it is soluble or part of a matrix in the cell microenvironment, and underscores the importance of studying matrix components in a manner that closely resembles what cells encounter in nature.

We also present evidence indicating that CD44 interactions with HA affect the composition of the stroma. We show that CD44/HA interactions do not affect the transcriptional activity of MMP9 in breast tumor cells seeded on type I collagen, as was previously shown in osteoclast-like cells [58]. However, while CD44/HA interactions do not seem to affect MMP9 activity in collagen I gels, we show that in collagen IV matrices containing HA, cells employ the use of MMP9 as they invade when CD44 can no longer bind HA.

The basement membrane surrounding the ducts of the mammary gland is essential for the maintenance of tissue localization. Degradation of this matrix can lead to breast cancer cell invasiveness and ultimately enable metastasis. Here we demonstrate that CD44 binding to HA present in the context of a collagen IV matrix inhibits MMP9 activity. Breast cancer cell invasion is inhibited into collagen IV gels when HA is present in the matrix. However, if CD44 activity is inhibited by BRIC 235, invasion is restored even in the presence of HA in an MMP9 dependent fashion. In the absence of HA, invasion into collagen IV gels does not seem to be MMP9 dependent. This indicates that while CD44 interactions with HA may decrease MMP9 activity, and the invasiveness of breast cancer cells, in the absence of CD44, HA may increase invasiveness through an MMP9 dependent mechanism. It may be possible that in the absence of CD44, HA may be binding to an alternate receptor such as RHAMM, to mediate increased invasion and increased MMP9 activity.

As demonstrated previously, HA mediated activities depends on the context in which HA is presented to the cell. Previously we showed differential HA mediated activity depending on whether HA was found soluble in the media or immobilized in a matrix. However, here we show evidence that it is not only the solubility of HA that matters for its activity, but also which matrix HA is found in. Here we show

MMP9 activity is not affected by HA in collagen I gels. However, when HA localized in a collagen IV matrix, HA inhibits MMP9 mediated invasion in a CD44 mediated fashion. It is tempting to speculate that differential HA mediated activity may be occuring as a result of CD44 binding to both HA and collagen. While many different ligands for CD44 have been identified, including collagen, studies have focused mainly of HA mediated activities and not much is known about the effects of collagen on CD44 signaling. The HA binding site, known as the 'link module' is located in at the amino-terminus end of the extracellular domain. It is tempting to speculate that the stalk of the extracellular domain of CD44 may contain a collagen binding site that can affect HA signaling depending on which collagen CD44 is binding.

Remodeling of the extracellular matrix is extremely important to a metastasizing cell. Studies examining migrating cells in traditional 2D systems may not be fully elucidating all of the interactions necessary for a cell to move through a 3D tissue. Tissue microarchitecture is a complex construction comprised of various components that may function together rather than individually. By isolating matrix components and adding them individually to cells, we cannot fully understand how matrix components affect each other and how their additive effects modulate cellular morphology. This study brings to light these shortcomings as it indicates that even when studying matrix components in 3D matrices, such as collagen or HA, additional elements with the matrix may further change phenotypes. This underscores the necessity to examine epithelial stromal interactions in a context as similar as possible to that seen *in vivo.*

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